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XRF and XAFS Analysis of Electrophoretically Isolated Nondenatured Proteins

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Abstract

Microbial metal reduction is relevant for developing bioremediation strategies for contaminated sites containing toxic metal compounds, but the molecular mechanisms involved and the regulation of those mechanisms are poorly understood. Metal reduction capability is microbe specific, yet the involvement of metalloproteins in the transport of electrons from electron donor molecules to electron acceptor molecules (i.e., to the metals to be reduced) is common to all metal-reducing organisms. A thorough understanding of the mechanisms involved in metal reduction by microbes, with a focus on their metalloproteins, will provide valuable information about the metal reduction capability of the organisms and could provide general insight into the mechanisms of metalloprotein expression and regulation in other microbial systems as well. We are developing methods for the efficient separation, detection, and quantification of metalloproteins. Electrophoretic methods for protein separation have been combined with X-ray fluorescence mapping and XAFS to determine the abundance and local chemical environment of iron within a cytochrome.

1. Introduction

Microbial metalloproteins mediate critical processes such as metal reduction, tolerance to radiation, and degradation of organic contaminants. Elucidating the involvement of metalloproteins in biochemical networks is crucial to the development of bioremediation strategies for waste sites containing toxic organic and metal compounds, because currently the molecular mechanisms involved in microbial metal reduction and the regulation of those processes are only partially understood. To elucidate metalloprotein expression within whole cells and microbial communities, we have begun developing new technologies and strategies to enable coupling of X-ray fluorescence (XRF) imaging, XRF elemental analysis, and XAFS with electrophoretic methods.

2. Metalloproteins

Metalloproteins have been estimated to represent one-third of the proteins synthesized by biological systems. As the name suggests, these proteins include a metal atom or atoms in their three-dimensional structures. Typically, one or more relatively small metal atoms are associated with a much larger protein molecule. Metalloproteins participate in many of the essential reactions of metabolism and energy production, including reactions with potential bioremediation applications such as reduction of toxic metals and radionuclides and degradation of hazardous organic contaminants. In respiratory energy production, metalloproteins pass electrons down a controlled thermodynamic gradient to a final electron acceptor by altering the valence states of the metals. In the absence of oxygen, organisms can produce metalloproteins essential for the reduction of available metal ions, including contaminant metals found in soils, sediments, and aquatic systems. The metalloproteins that mediate these respiratory electron transfers are primarily cytochromes that carry Fe atoms coordinated in porphyrin rings. Other metals, notably Cu and Mn, also participate in such processes. For example, the terminal step of oxygen-dependent respiration involves cytochrome c oxidase, an enzyme containing Cu and Fe.

Beyond metal reduction, metalloproteins from various organisms also catalyze the oxidation of chelating agents (thereby halting dispersal of heavy metals and radionuclides mobilized by them) and the degradation of organic pollutants. Metalloproteins are also undoubtedly involved in microbial processes controlling the fixation and general cycling of carbon.

Current methods for identifying and characterizing new metalloproteins in complex mixtures are slow and laborious. Isotopic labeling of proteins with specific metal tags enables detection only of metalloproteins containing that specific metal (e.g., Fe). *In situ* labeling with radiolabeled metals can be problematic if the cell's uptake and metabolism of the radiolabeled molecules are inefficient. Staining methods for detection of metalloproteins, such as the heme stains, also allow for the detection of only one type of metalloprotein. The sensitivity of these specialized stains is also limited, necessitating the use of relatively large amounts of proteins (often over 100 μg of pure protein) for detection. New methods must be developed, therefore, to achieve high-throughput, global discovery and characterization of individual metalloproteins of different types in the complex mixtures of proteins produced by organisms.

3. Electrophoretic Separation of Proteins under Nondenaturing Conditions

The global detection and characterization of metalloproteins requires, in addition to the detection and characterization methods provided by XRF and XAFS, a method for separating complex mixtures into distinct protein components. Although many metalloproteins retain their metal moieties under denaturing conditions, we hypothesize that separation under nondenaturing conditions will preserve even the protein-metal interactions that might be disrupted by denaturation.

Separation of native proteins (i.e., nondenatured functional proteins with minimally disrupted protein structure) by one-dimensional (1-D) gel electrophoresis in cellulose acetate, starch, or polyacrylamide has been used effectively to characterize

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hundreds of enzyme activities and isozyme polymorphisms [1, 2]. Such 1-D separations of nondenatured proteins can be achieved by using 1-D zone electrophoresis or isoelectric focusing in starch or polyacrylamide. For zone electrophoresis, buffer and gel conditions are selected to optimize differences in the mass-to-charge ratio of the sample proteins. Selection of the ionic strength and pH of the running and sample buffers influences the charge separation, while selection of the separation matrix concentration, which influences the final pore size of the gel, affects the mass separation. Separation by isoelectric focusing, in contrast, depends only on differences in the net charges of the proteins, independent of their masses.

A 2-D separation based on two independent parameters, analogous to the separation by isoelectric point and size used in denaturing 2-D electrophoresis (2DE), would obviously overcome co-migration of proteins and dramatically increase the number of distinct proteins detectable. We recently developed such a method for the 2-D separation of proteins under nondenaturing conditions and applied the method to the analysis of proteins from the metal-reducing microbe *Shewanella oneidensis* [3].

4. Detection of Metalloproteins in Polyacrylamide Gel Strips

Although the XRF technique is a very powerful probe for identifying a variety of elements in a microbial system, the information provided is only the average for all of the free metal ions and the metalloproteins at the point of interaction with the X-ray beam. To investigate the interaction between a metal and one of the specific proteins expressed by the microbe, the size of the X-ray probe must be comparable to or smaller than the dimensions of the spatially resolved protein of interest. For the 1-D and 2-D gel systems discussed here, 0.1- to 1.0-mm X-ray beams are required to analyze the resolved protein bands in the polyacrylamide gels. Production of high-intensity X-ray beams of this size with an undulator X-ray source has been accomplished simply with the use of apertures the size of the desired spot.

In a preliminary experiment, a sample containing 0.5 μg of cytochrome c was resolved by using isoelectric focusing in immobilized pH gradient (IPG) strips. The portion of the gel containing the protein was cut out and mounted on a sample holder compatible with existing mounting devices at the Materials Research Collaborative Access Team (MRCAT) insertion device beamline at the Advanced Photon Source (APS) [4]. With a single-element solid-state XRF detector, a strong Fe Kα fluorescence signal was easily detected after less than 10 s of irradiation with a 10.5-keV X-ray beam. This result demonstrated that the necessary sensitivity exists for detection of the metal atoms in metalloproteins resolved in gels. In a subsequent experiment, various amounts of catalase (20, 100, and 200 μg) were resolved by isoelectric focusing on replicate IPG strips. One strip was stained for protein, and the others were then "imaged" in 1-D by XRF by translating the strip through the X-ray beam. Normalized integrated intensity of the Fe Kα fluorescence at the protein location on the strip is plotted versus the mass of metalloprotein applied to the IPG strips in Fig. 1. Results of linear regression analysis of these data conservatively indicate a 4 μg detector, 20-s integration time, and 10-keV X-ray probe energy. Realistic improvements to the experimental setup for better detection sensitivity include (1) measurement near resonance for Fe fluorescence detection (factor of ∼2), (2) use of all 13 elements of the solid-state detector at the MRCAT beamline (factor of 3.6), and (3) increased data collection time from 20 s to 600 s

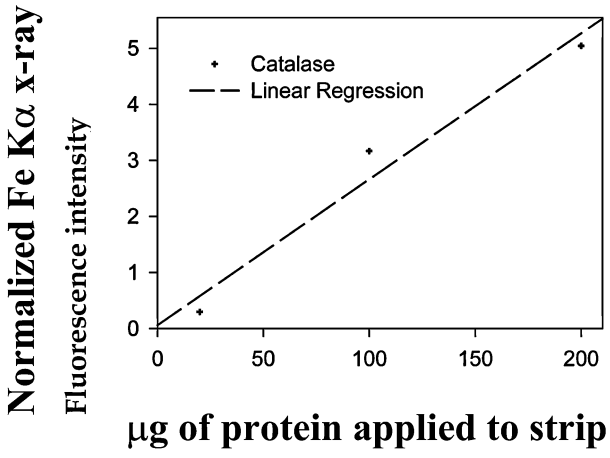

Fig. 1. Normalized integrated Fe Kα fluorescence intensity at the protein location on the IPG strip versus the mass of metalloprotein applied.

(factor of 30). These improvements would result in increased elemental detection sensitivity of >200-fold for a metalloprotein similar to catalase. This translates to sensitivity of better than 20 ng for metalloproteins with concentrations of Fe similar to that of catalase.

We evaluated this technique for investigating samples from microbial cultures. Specifically, we compared the reproducibility and accuracy of XRF elemental mapping for identifying the presence of cytochrome c7 (a triheme Fe-centered metalloprotein) overexpressed by an *Escherichia coli* culture (100 μg of total protein). In addition, 50 and 100 μg of purified cytochrome c7 (as calibration standards) and 200 μg of material from an *E. coli* control culture (i.e., not overexpressing cytochrome c7) were also measured. XRF measurements of the spatial distribution of the Fe Kα fluorescent intensity on a 1-D gel were performed at the MRCAT [4] beamline. A 10.5-keV X-ray beam (0.77mm × 1.0 mm) was used as a probe, and the fluorescent X-ray intensity was monitored with three elements of a multielement solid-state detector. Measurements were made on all samples at room temperature and at atmospheric pressures. Results for the relative intensities of the Fe Kα fluorescence radiation relative to the position on the gel are shown in Fig. 2. These results clearly illustrate (1) correlated elevations in Fe Kα fluorescence intensity for the cytochrome c7 standard and the *E. coli* overexpressing cytochrome c7 (at ∼59,000 μm, marked "A" on the strip),

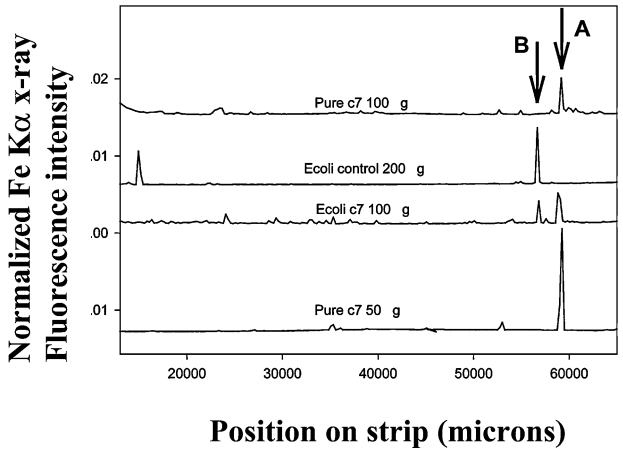

Fig. 2. Relative Fe Kα X-ray fluorescence intensities for cytochrome c7 standards and *E. coli* cell lysates relative to their positions on 1-D IPG gels.

(2) reproducibility (i.e., collocation of the Fe peak at ~59,000 μm, marked "A" on the gel strip, for two replicate samples), and (3) the presence of additional, reproducible Fe fluorescence radiation in both the overexpressed cytochrome c7 and control *E. coli* samples (at ~57,000 μm, marked "B" on the gel strip). These results clearly demonstrate that this approach enables identification of overexpressed metalloproteins in cultures. Observation of Fe peak at ~57,000 μm in both types of *E. coli* samples further demonstrates that this technique does not necessarily require a metalloprotein to be overexpressed in order to be detected.

5. XAFS Analysis of Proteins Separated on Nondenaturing Gels

In many instances, although identification of the presence of metalloproteins is extremely valuable, additional information concerning the structure and function of the metal center of the metalloprotein is desirable. The atoms that coordinate the metals of a protein alter their chemistry. For example, in cytochromes, Fe is bound by four equatorial nitrogen atoms from the porphyrin ring, plus axial ligands from the protein or the solvent. In some cases, a sulfur atom provides the axial ligand. XAFS spectroscopy can be an extremely valuable tool for probing the local chemical and structural environment of the metal within metalloproteins. To investigate the possibility of coupling the XAFS technique to procedures discussed above, we have made Fe XAFS measurements on a catalase protein after isoelectric focusing on polyacrylamide on a plastic backing (IPG strip). XAFS data were collected at room temperature in the fluorescence mode with an ion chamber in the Stern-Heald geometry [5, 6] by using a Lytle detector with an Mn filter. Linearity tests [7] indicated less than 0.5% nonlinearity in the experimental setup for a 50% attenuation of the incident radiation. Incident and transmitted X-ray intensities were monitored with ionization chambers with 100% free-flowing nitrogen gas at atmospheric pressures. Results of the fitting of the data ($\Delta k = 2.5$–$8.0\,\text{Å}^{-1}$, $\Delta r = 1$–$3\,\text{Å}$, 7 floating variables, and 2 degrees of freedom) to theoretical standards generated with the FEFF program [8] are shown in Fig. 3. Results from fitting analysis of these data indicate an average of 4 N/O and 1 N/O (at 1.98 Å and 2.05 Å, respectively) and 8 carbons (at 3.05 Å), plus additional carbons and multiple-scattering effects (at 3.2–3.4 Å), contributing to the local environment of the Fe. These results are consistent with previous XAFS studies of other purified and concentrated catalase proteins [9–11].

For future work, a low-temperature sample holder will be designed to reduce radiation effects that might alter the proteins being measured with XAFS. To increase the sensitivity of the XAFS technique to lower concentrations of metalloproteins, future XAFS measurements will also be made with a 13-element solid-state fluorescence detector.

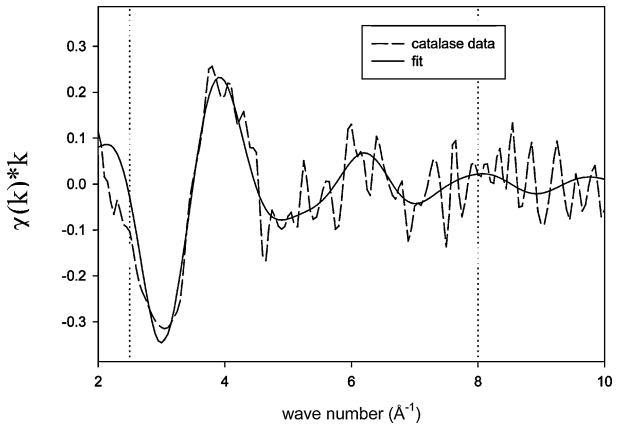

Fig. 3. Theoretical fitting (dashed line) of XAFS chi data (solid line) from catalase spatially isolated on an IPG strip.

6. Summary

In summary, we have described the results of the development of new approaches to identify and characterize metalloproteins. Specifically, we have described the integration of XRF mapping, XRF elemental analysis, and XAFS with electrophoretic methods. Further development of these integrated techniques to enable high-throughput analysis of frozen samples (to reduce the effects of radiation damage) holds great promise for the investigation of proteomic expression in a wide variety of biological systems.

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